1978

Amino acid analysis and technical aspects of gel electrophoresis on vitellin from Aedes aegypti (L.) the yellow fever mosquito.

Gail Vanessa Willard
University of Massachusetts Amherst

AMINO ACID ANALYSIS AND TECHNICAL ASPECTS OF GEL ELECTROPHORESIS ON VITELLIN FROM *AEDES AEGYPTI* (L.) THE YELLOW FEVER MOSQUITO

A Thesis Presented

By

GAIL VANESSA WILLARD

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

June 1978

Entomology

AMINO ACID ANALYSIS AND TECHNICAL ASPECTS OF GEL ELECTROPHORESIS ON VITELLIN FROM *AEDES AEGYPTI* (L.) THE YELLOW FEVER MOSQUITO

A Thesis Presented

By

GAIL VANESSA WILLARD

Approved as to style and content by:

James B Kring

Dr. James B. Kring, Chairman of Committee

John D. Edman

John D. Edman

Larry K. Cole

Larry K. Cole

James B Kring

Dr. James B. Kring, Department Head
Entomology

ACKNOWLEDGEMENTS

First, I would like to express my sincerest gratitude to Drs. James B. Kring, John D. Edman, Larry K. Cole and Henry H. Hagedorn for their help, guidance and encouragement during this project and preparation of this thesis. I would also like to thank Walter Kaczor who also gave me support.

Next I would like to express my appreciation to Dr. Peter Pellet of the Department of Food Science and Nutrition, who allowed me use of his amino acid analyzer and also to Louis Raboin for the many hours he spent assisting me in the preparation and running of my samples. I need also to thank Curtis Fullmer of Cornell University for running an amino acid analysis.

Finally I would like to thank Eric Johnson for his encouragement, support and general belief in my ability to complete the tasks set before me. Through the whole ordeal he never let me forget that "with God all things are possible."

TABLE OF CONTENTS

ABSTRACT v

INTRODUCTION 1

LITERATURE REVIEW 4

General Background 4
Isolation and Gel Electrophoresis Methods 4
Methods of Amino Acid Analysis 9

FLOW CHARTS OF PROCEDURES 14

MATERIALS AND METHODS 18

Isolation of Vitellin 18
Gel Electrophoresis of Vitellin 20
Amino Acid Analysis of Vitellin 21

RESULTS AND DISCUSSION 22

General Introduction 22
Isolation, Purification and Gel Electrophoresis of the Vitellin 23
Amino Acid Analysis 33

FIGURES AND TABLES 36

SUMMARY AND CONCLUSIONS 43

General Introduction 43
Isolation, Purification and Gel Electrophoresis of the Vitellin 43
Amino Acid Analysis 45

REFERENCES CITED 47

APPENDICES 51

ABSTRACT

A method is presented for isolating and purifying vitellin(s) from the mosquito Aedes aegypti so that consistent results could be obtained. The question as to whether vitellin is a single protein or a complex of related proteins is discussed. This thesis also shows the need for the use of recrystallized acrylamide when performing gel electrophoresis. Moreover, it is also necessary to maintain consistent parameters when electrophoresing the vitellin. Amino acid analysis of Aedes aegypti vitellin was carried out, and comparisons made to the analysis of other insect vitellins. These results demonstrated that Aedes aegypti vitellin is closely related in amino acid composition to the other insect vitellins examined.

INTRODUCTION

Insects are the most prolific and most numerous of all animals, yet little is known about their reproductive processes. Biting insects present many problems to man, one of the most serious being the vectoring of diseases. Strategies have been suggested to control these vectors, among them being the regulation or disruption of their reproduction. However in order to formulate and exploit such methods, there first is a need to fully understand the insect's reproductive processes.

In 1954 it was demonstrated in the Cercropia silkmoth that female specific protein was transferred during oogenesis from the hemolymph to the eggs (Telfer, 1954). Such yolk precursor proteins or vitellogenins (Pan et al., 1969) have since been found to occur generally in the hemolymph of adult female insects. They are deposited in the yolk, in a form that has been termed vitellin (Wyatt and Pan, 1978), since there may be modifications in the hemolymph precursors in some cases (Chen et al., 1976; Kelly and Telfer, 1977). In Aedes aegypti, the experimental animal used in this research, vitellin and vitellogenin have the same antigenic qualities and molecular weights (Kelly and

Telfer, 1977; Hagedorn et al., 1978, unpublished data) and therefore are essentially the same.

Vitellin is usually isolated and concentrated by extraction in a high salt buffer, followed by precipitation in a buffer of low ionic strength (Dejmal & Brookes, 1972), however within this technique there is a great deal of variability. Vitellin from various insects has been isolated by means of a wide variety of buffer systems (Dejmal and Brookes, 1972; McGregor and Loughton, 1974; Hagedorn and Judson, 1972; Hagedorn et al., 1978). Nonetheless, it is the ionic strength and pH of the buffer used (Oie et al., 1975), that seems to have the most effect on the percent recovery, and purity of the vitellin isolated. This fact seems to have been overlooked at various times by different investigators and might account for some of the variance in the results.

As much variability as exists in the methods of isolation, there is probably an equal lack of uniformity in the methods of gel electrophoresis (Weber and Osborn, 1969; Orstein and Davis, 1962; Kane, 1973; Laemmii, 1970). However the overall method chosen does not seem to have as much bearing on the results as does the controlling and standardizing of the internal factors within a given system such as pH of the buffers, amount of sample applied to each gel, amperage used when running gels and the running temperature.

Amino acid analysis of insect vitellin is in a formative state. To date the amino acid composition of only eight insects has been examined (Kunkel and Pan, 1976; Chino et al., 1977; Kambysellis, 1977). No amino acid analysis has been done with any nematocerous Diptera, so no comparison can be made between this group and other insect groups.

The first objective of my research was to refine the method for isolating and purifying vitellin so consistently reproducible results could be obtained. I also wanted to improve the technique for electrophoresing the vitellin so the results could be standardized and direct comparisons could be made between experiments. These results were also necessary to discuss whether vitellin consisted of one or more than one protein.

The second objective of this research was to prepare and carry out an amino acid analysis of vitellin from an anautogenous mosquito and to compare these results with those obtained in the analysis of other insects.

LITERATURE REVIEW

General Background

In the anautogenous mosquito, ecdysone produced by the vitellogenic ovary (an ovary stimulated by EDNH) stimulates the fat body to release vitellogenin into the hemolymph (Hagedorn, 1974; Hagedorn et al., 1973). The vitellogenins subsequently enter the oocytes by pinocytosis (Roth and Porter, 1964) where they become 20 - 30 times more concentrated. Other proteins are largely excluded (Telfer, 1960). Once the vitellogenin has been deposited in the ovary it is termed vitellin (Wyatt and Pan, 1978).

Isolation and Gel Electrophoresis Methods

Vitellin was first isolated from Leucophaea madarae by Dejmal and Brookes in 1968. The ionic strength of the NaCl solution was shown to be a critical factor in the solubilization of the yolk protein. In 1972 they suggested extracting the vitellin in a high salt solution, followed by precipitation at a low ionic strength to purify the vitellin, a technique which has often proved useful (Wyatt and Pan, 1978). Hagedorn and Judson (1972) extracted vitellin from Aedes aegypti using Tris-chloride buffer of high

ionic strength, instead of just a salt solution as used by Dejmal and Brookes. In 1974 Pan and Wallace used a more complex Tris citrate buffer system, to purify vitellin from Hyalophora cercropia. Oie et al., (1975) who isolated and purified vitellin from Blatella germanica, compared buffers of various pH and salt concentrations for their ability to extract vitellin. They showed that in B. germanica no detectable amount of vitellin was solubilized when ovaries were extracted with a 0.1 or 0.2 M NaCl solution, where as a 0.4 or 0.8 M NaCl solution precipitated large amounts of vitellin from the ovaries. The pH of the extractant also proved to be a critical factor when solubilizing vitellin. Buffers of pH of <7 did not effectively solubilize the vitellin, while buffers of pH in the range 7 - 9 were quite effective. Furthermore, they were able to show that with a buffer in which favorable conditions of salt concentration and pH were combined the extraction efficiency did not appreciably improve. However, the use of a buffered system combining optimal pH and salt concentration did improve the percent recovery of vitellin in later purification steps.

Dejmal and Brookes (1968) found that lowering the ionic strength of saline extracts to 0.2 M NaCl by dilution with water readily precipitated a major fraction of the soluble proteins (which was 80% vitellin) in Leucophaea

madarae. In B. germanica however, Oie et al. (1975) found that precipitation of the proteins by dilution with water required a far larger volume of water than suggested by Dejmal and Brookes. Moreover, they found that precipitation still proceeded so slowly, so it was impractical for them to adopt this procedure of purification. Instead, a more efficient precipitation was induced by lowering of the pH of the extract to below pH 7. This treatment readily precipitated the bulk of the protein and was used as a purification step. McGregor and Loughton (1977), when isolating Locusta migratoria vitellin, found that after solubilizing it in 0.8 M tris buffer the vitellin could be recovered by adding 10 volumes of ice cold distilled water, followed by centrifugation. Gellissen et al., (1976) also used a technique similar to Dejmal and Brookes (1968). They solubilized L. migratoria vitellin in 0.4 M NaCl (unbuffered), then precipitated the vitellin by lowering the ionic strength of the solution by dilution and isolated by centrifugation.

Chino et al., (1977) solubilized Philosamia cynthia vitellin in a 0.02 M phosphate buffer with 0.125 M KCl; this has a lower salt concentration and lower pH (pH 6) than other buffer systems used. Despite this fact, they were able to solubilize and later recover the vitellin with this method, though no comment was made as to the percent of recovery. No rationale was given for the use of the homogenizing-solubilizing buffer with the lower pH and salt

concentration. Hagedorn et al., (1978) used a technique similar to both Dejmal and Brookes (1972) and Oie et al., (1975). They solubilized their vitellin in a 0.05 M Tris-phosphate buffer, pH 8.0 containing 0.25 M NaCl, then precipitated it by dialyzing against a Tris-phosphate buffer (ph 6), without salt, thus reducing both the pH and the salt concentration to precipitate the vitellin.

Solubilization and precipitation procedures usually only partially purify the vitellin so further purification steps are quite often used. Ammonium sulfate precipitation is probably the easiest method since it does not require the use of a column, but it also provides the lowest degree of purification (Oie et al., 1975). Gingeras et al., (1973) and Kambysellis (1977) modified this technique and used selective ammonium sulfate precipitation to isolate vitellin from Drosophila sp. which improved somewhat on the purity of the vitellin. After the vitellin had been precipitated, they further increased its purity by solubilizing it in a Tris buffer (pH 8.2) and then running it through either a preparatory electrophoresis or a Sepharose 4B column (Gingeras, 1976). Chino et al., (1976, 1977) used a 75% ammonium sulfate solution to precipitate the vitellin from P. cynthia, then dissolved it in distilled water and dialyzed against distilled water. After dialysis, 10 volumes of distilled water pHed to 6 were added to precipitate the vitellin,

it was subsequently solubilized in a phosphate buffer and run through a DEAE column as the final purification step.

Upon surveying the various purification techniques it is evident that diethylaminoethyl cellulose (DEAE) column chromatography is most often used and the most effective. Oie et al., (1975) in comparing purification techniques, found that DEAE-cellulose gave the highest degree of purity as well as a good yield of the vitellin. Hagedorn and Judson (1972) used a combination of Sepharose and DEAE columns which seemed to be just as effective as using only a DEAE column (Gellisen et al., 1976; Hagedorn et al., 1978).

Other approaches have been used in purifying vitellin. Englemann et al., (1976) used a DEAE column and then further purified L. madarae vitellin by using a cesium chloride gradient. This yielded an extremely pure sample of vitellin. At the other extreme McGregor and Loughton (1977) who used only a Sepharose 6B column to purify vitellin from L. migratoria.

It appears from the literature that each investigator has his or her own "favorite" gel electrophoresing technique and there seems to be no effort toward standardization. The Laemmli systems (Laemmli, 1970) gives extremely high resolution. Complete cellular extracts can be applied without smearing. Samples as large as 1 ml, containing considerable salt, can be analyzed. This method however, is

not as reliable as the Weber and Osborn (1969) method for determining molecular weights (Scrader and O'Malley, 1977).

Variations on the original Orstein and Davis technique (1962) are frequently used (Chino et al., 1976; Chino et al., 1977; Oie et al., 1975; Englemann et al., 1976; Chino et al., 1977) along with the Weber and Osborn technique also being used quite extensively (Kunkel and Pan, 1976; McGregor and Loughton, 1977; Chino et al., 1977; Chapman et al., 1975). Some investigators design their own buffer system to suit their individual needs as did Whitmore and Gilbert (1974).

Methods of Amino Acid Analysis

In amino acid composition, a number of insect vitellogenins and vitellins show a common pattern as characterized by high aspartic and glutamic acid.[1] This pattern remarkably resembles the amino acid composition of some lipovitellins of birds and amphibia, while differing somewhat from that of some non-vitellogenic insect plasma proteins (Wyatt and Pan, 1978; Kunkel and Pan, 1976; Chen et al., 1976).

---

[1]When aspartic and glutamic acids are referred to they are actually asparagine and asparate, and glutamine and glutamate respectively; since in most analyses they are not separated. Due to this fact, it would not seem unusual that these valves would be higher than the other values.

To date amino acid analysis have been done on seven insect vitellins (eight, if Drosophila melanogaster and D. virilis are counted separately). The first analysis was done by DeLoof and DeWilde in 1970 on the Colorado Potato beetle (Leptinotarsa decemlineata). They only did acid hydrolysis so their values for threonine, serine, cysteine, tyrosine and tryptophan were abnormally low. Still, they found the high percent of glutamic acid and aspartic acid, which became a characteristic trend in other insect vitellins.

The next amino acid analysis was done in 1972 by Dejmal and Brookes. They analyzed vitellin from L. madarae, but did little except present the data in tabular form. In 1974 Englemann and Friedel also did an amino acid analysis of the L. madarae protein. They examined both the vitellogenins from the hemolymph and the vitellins from the ovaries. They did a complete hydrolysis and used a Beckman 120B Amino Acid Analyzer (as did Dejmal and Brookes) to do the analysis. They also found glutamic and aspartic acid in extremely large amounts with leucine, serine and valine also on the high side.

In 1976 analysis was carried out with B. germanica and H. cercropia, and comparisons made between insect, avian and amphibian vitellins, and non-vitellins (Kunkel and Pan, 1976). Aspartic and glutamic acids were high in all categories, but the vitellins did vary somewhat from non-vitellins in their overall amino acid compositions.

Kunkel and Pan concluded that vitellins did resemble each other as a loose group, but even with this resemblance there were no immunological cross-reactions outside of the generic level among cockroaches (Kunkel and Pan, 1976) or outside of the family level in saturniid silkmoths (Telfer, 1954; Laufer, 1964). Kunkel and Pan were able to show conclusively that Dictyoptera and Lepidoptera will not support vitellogenesis in each others oocytes even though they have very similar gross chemical properties. These results indicate that the selective uptake mechanism involves recognition of subtle differences in the protein structure. This suggests marked divergence in amino acid sequence despite similarity in net composition (Wyatt and Pan, 1978).

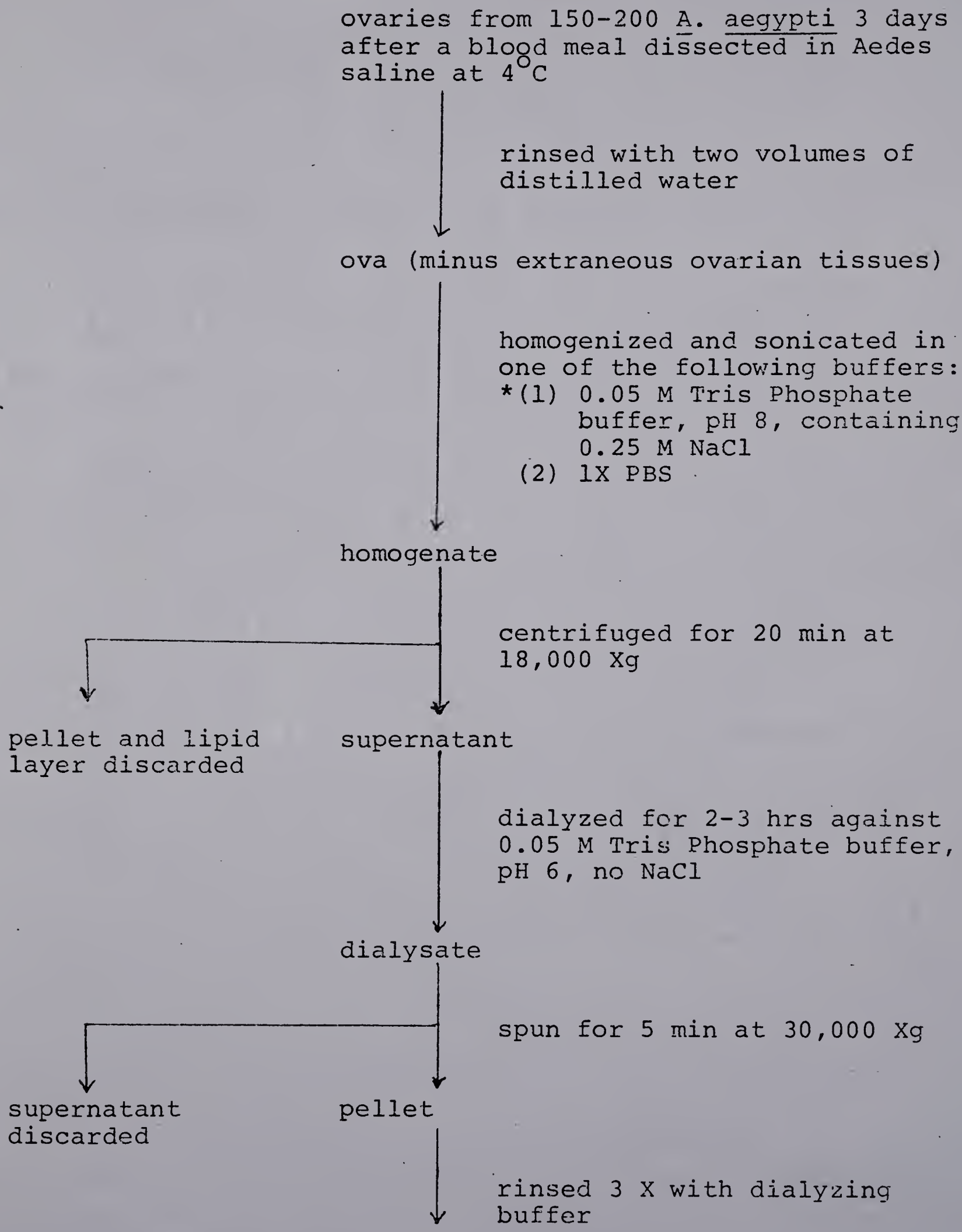
Three amino acid analysis of insect vitellins were done in 1977. Gellissen et al., (1977) showed that L. migratoria had the expected high aspartic and glutamic acids. They also found a low half cysteine content which also is characteristically low in the other vitellins. McGregor and Loughton (1977) also did an analysis of the L. migratoria vitellin. They found an abnormally high glycine count along with the usually prominent aspartic and glutamic acids. This discrepancy could be related to the fact that glycine was probably used in their buffer system (Kambysellis, 1977). Chino et al., (1977) did an amino acid analysis of vitellin from P. cynthia. Like Engelmann and Friedel (1974) they

analyzed both the hemolymph vitellogenin and the ovarian vitellin. From their analytical data and other information, they were able to conclude that both vitellogenin and vitellin are the same protein in P. cynthia. Kambysellis (1977), modified from Gingeras (1976), compared the amino acid composition of purified yolk protein from both D. melanogaster and D. virilis. He found the percent of glycine to be extremely high (similar to McGregor and Loughton, 1977). He attributed this to the possibility that some of the glycine from the Tris glycine buffer system contaminated the sample even though the purified protein was exhaustively dialyzed against distilled water. He found high amounts of glutamic and aspartic acids, but in D. virilis serine was higher than both glutamic and aspartic acids. He also made the suggestion that because cysteine was more abundant in D. virilis than in D. melanogaster vitellin, D. virilis vitellin had a more compact configuration. This comment might be viewed with skepticism since the amounts of cysteine usually cannot be accurately determined.

In order to meaningfully compare the various amino acid analysis of insect vitellogenins and vitellins an appropriate quantitative method was needed. Kunkel and Hagedorn (1978, unpublished manuscript) suggested the use of the SΔQ and Manhattan distance (MD) metric for comparing amino acid compositions. The SΔQ method emphasizes those amino acids that differ the most between two proteins since

it squares each difference; it is most appropriate when looking for differences between proteins. The MD method, which simply adds differences, is more appropriate for comparing the degree of difference between proteins, and has been described as useful for tree building (Kunkel and Hagedorn, 1978 unpublished). This would indicate that the SΔQ method is best for comparing vitellins and vitellogenins, while MD would probably be more useful in comparing vitellins and non-vitellins from different insects.

Flow Chart of Isolation, Purification and Gel Electrophoresis of Vitellin From Aedes aegypti



rinsed pellet

↓

solubilized in one of the following buffers:
*(1) 0.01 M Na-K phosphate buffer, pH 7, no NaCl
(2) 0.1 M Na-Na phosphate buffer, pH 7, no NaCl

↓

extractant

↓

aliquot saved to check purity of precolumn vitellin by gel electrophoresis

solution rinsed onto a prewashed Whatman DEAE column with solubilized buffer

↓

O.D. at 280 of wash from column monitored until background levels was reached

↓

vitellin eluted off the column with one of the following buffers:
*(1) 0.1 M Na-K phosphate buffer, pH 7, 0.25 M NaCl
(2) 0.1 M Na-Na phosphate buffer, pH 7, 0.25 M NaCl

↓

eluent containing vitellin

↓

For amino acid analysis, the eluent was processed as diagrammed in "Flow Chart of Amino Acid Analysis of Vitellin" on page 17

↓

sample mixed with a 0.1% Bromophenol Blue-10% sucrose solution in a 20:1 ratio of sample to total solution

↓

vitellin sample was dialyzed overnight against sample buffer

↓

Dialysate is boiled for 6 min (or not boiled for a control)

↓

sample mixed with a 0.1% Bromophenol Blue-10% sucrose solution in a 20:1 ratio of sample to total solution

↓

applied to a 6% acrylamide gel containing either *recrystallized or unrecrystallized acrylamide in varying concentrations

↓

gels electrophoresed for 20 hrs at 1 Ma/tube at 4°C

↓

gels removed from tubes

↓

gels fixed and stained

applied to either a *7.5% or 5% acrylamide gel 1% SDS containing either recrystallized or unrecrystallized acrylamide in varying concentrations

↓

gels electrophoresed for 12 hrs at 1 mA/tube at room temp. (~23°C)

↓

gels removed from tubes

↓

gels fixed and stained

---

*Buffers and procedures that gave the most consistent, efficient results in the systems.

Flow Chart of Amino Acid Analysis of Aedes aegypti Vitellin

Purified sample collected in eluent

↓ dialyzed overnight against 0.05 M Tris Phosphate buffer, pH 6, no NaCl

dialysate collected

↓ freeze-dried

sample collected and weighed

↓ diluted in 1-2 mls of 6N HCl

solution sealed under a vacuum

↓ hydrolyzed for 22 hrs at 110 C

hydrolysate recovered

↓ freeze-dried

sample dissolved in 1-2 mls sodium citrate buffer, pH 2.2

↓

sample analyzed on a 120C or 119C Beckman Amino Acid Analyzer

MATERIALS AND METHODS

Isolation of Vitellin

Larvae of *Aedes aegypti* were reared in groups of 200 in plastic trays containing 450 ml of distilled water at 27°C. They were fed daily on a 1:1:1 mixture of finely ground rat chow, lactoalbuminhydrolysate and dry Brewer's yeast (Day 1 = 400 mg, Day 2 = none, Day 3 and 4 = 400 mg, Day 5 and 6 = 600 mg). Adults were allowed continuous access to a 3% sucrose solution.

Ovaries from 150-200, 3-5 day old mosquitoes were removed 3 days after being fed on a rabbit (mosquitoes were anesthetized on ice) and stored for a maximum of 24 hours in Aedes saline (Appendix A) at 4°C (all subsequent preparative manipulations also were conducted at 4°C). Ovaries were washed three times in 5 volumes of distilled water to release the ova and remove extraneous ovarian tissues by decanting. Ova were homogenized with a Pyrex ground glass homogenizer (No. 7727) in 2 volumes of a phosphate buffer of high ionic strength (Appendix B), sonicated on a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc.) for 4 minutes and centrifuged at 18,000 g in a Sorvall centrifuge with a swinging bucket rotor at 4°C for 20 minutes. Supernatant

was removed with a pasteur pipet (avoiding the top lipid layer) and dialyzed for 2-3 hours in a 250 ml graduate cylinder with a stir bar against ~100 volumes of no NaCl, Tris-phosphate buffer (Appendix B) with 1 change of buffer. Dialysate was removed with a Pasteur pipet, centrifuged at 3,000 g in a Sorvall Centrifuge with a swinging bucket rotor for 5 minutes, and the resulting pellet was rinsed several times by suspending it in 2 volumes of no NaCl, Tris-phosphate buffer, bringing it out of suspension on ice, then re-centrifuging under the same conditions as before. After being rinsed, the pellet was dissolved in 1 1/2-2 volumes of phosphate buffer (Appendix B). An aliquot (100-200 µl) was saved to check the purity of the precolumn preparation using gel electrophoresis. The remaining solution was run through an 8 x 45 mm DEAE column. The column's solid support was Whatman DEAE 52 (Cat. #24521) which had been swelled overnight in the phosphate eluting buffer (Appendix B) at room temperature (~23°C). The DEAE, suspended in the phosphate eluting buffer, was poured down a glass rod into a column which had a small plug of glass wool at the bottom. The DEAE was allowed to settle for 4-6 hrs under 1 volume of phosphate eluting buffer, then placed in the cold until it reached a temperature of 4°C. The column was rinsed with 50-100 ml of the phosphate eluting buffer. Sample was applied to the column and washed on with 25-35 ml of phosphate

eluting buffer. Fractions of 1 ml were continuously monitored at both 260 nm and 280 nm [1.55 x $O.D_{280}$ - 0.76 x $O.D_{260}$ = mg/ml (Hagedorn et al., 1978)].[2] When a level equal to that of the phosphate eluting buffer was reached, the phosphate eluting buffer containing 0.25 M NaCl was run through the column and the vitellin was eluted (modified from Hagedorn et al., 1978).

Gel Electrophoresis of Vitellin

Acrylamide gel solutions were made according to the Laemmli method (Laemmli, 1970; described in Appendices C & D) and poured into 8 mm x 120 mm electrophoresis tubes. To run the vitellin, acrylamide gels without sodium dodecylsulfate (SDS) were used. A sample of vitellin (20-150 µg) in the buffer which it was eluted from the column was mixed with a 0.1% Bromophenol Blue-10% sucrose solution in a 20:1 ratio of sample to total solution, and applied to the gels in varying concentrations. They were electrophoresed in a Buchler Instruments electrophoresis tank with Tris-glycine buffer (Appendix D) at 1mA per tube for 20 hours at 4°C. After electrophoresis the gels were removed from the tubes, fixed and stained (Appendices D and E).

To prepare the vitellin for SDS gels to examine the disassociation products of the vitellin, an aliquot of vitellin sample was dialyzed overnight in 5 volumes of BME

---

[2]Some samples with a high 280 nm absorption were added back to the column before eluting with the buffer containing 0.25 M NaCl.

solution (Appendix D) at 23°C. It is then mixed with 0.1% Bromophenol Blue-10% sucrose solution in a 20:1 ratio of sample to total solution, applied to the gels and electrophoresed at 1mA per tube for 12 hrs at approximately 23°C. After electrophoresis the gels were removed, fixed and stained (Appendices D and E).

Amino Acid Analysis

To do an amino acid analysis of the vitellin the eluted samples containing the vitellin were combined and dialyzed overnight against 100 volumes of 0.05 M Tris-phosphate buffer pH 6, (no NaCl)at 4°C, then freeze-dried. The remaining residue was weighed, suspended in 1-2 ml of 6 N HCl, sealed under a vacuum, then hydrolyzed for 22 hrs at 110°C. The hydrolysate was again freeze-dried, dissolved in 1-2 ml of 0.2 M sodium citrate buffer, pH 2.2 (modified from Chapman et al., 1975) and run on either a Beckman 120C Amino Acid Analyzer (which was performed by the lab of Dr. Peter Pellet, University of Massachusetts) or Beckman 119C Amino Acid Analyzer (which was performed by the lab of Curtis Fullmer, Cornell University).

RESULTS AND DISCUSSION

General Introduction

Due to the limited and incomplete information on Aedes aegypti vitellin and various other insect vitellins, it is difficult to immediately assume that vitellin is a single protein. To the best of my knowledge the total composition (proteins, lipids, carbohydrates and nucleic acids) of an insect ova has not been described or quantified. Given this and the methods of isolation and purification, that I and other investigators have used, assuming without reservation that the vitellin isolated is a single protein is questionable. Rather, it would seem more reasonable to consider two options, (1) that vitellin is a complex mixture of very related soluble proteins predominantly made up of yolk proteins (Telfer, 1960) or (2) that vitellin is a single, very complex yolk protein.

In Figure 7, the difference between chromatographed vitellin (Figures 7A and 7C) and non-chromatographed vitellin (Figure 7E) is minimal. Given the preparative steps to extract the soluble proteins before column chromatography (see Materials and Methods) it would seem unlikely that just a single protein would be extracted. Yet, if the appearance of the single vitellin band (Figure 7E) is interpreted

as a single protein, this would have to be the conclusion. Another point of interesting data is Figure 3D. This is vitellin eluted off the column with 0.1 M Na-Na Phosphate buffer, pH 7 containing 0.1 M NaCl, instead of the normal 0.25 M NaCl. This could be attributed to the fact that a portion of the sample didn't bind to the column or that vitellin is more than one protein and that one (or more) of the proteins from the "vitellin complex" is eluted at this molarity of salt. A third interesting observation is the very close correlation of the amino acid composition of A. aegypti vitellin with the amino acid compositions of other insect vitellins (Tables 1 and 2). This would seem to indicate that if vitellin is found to be a complex of closely related proteins in one insect system that there would be a good possibility that this would be the case in other insect systems, since vitellins obviously seem to be a closely related group.

Isolation, Purification and Gel Electrophoresis of the Vitellin

When I first attempted to obtain purified vitellin and perform gel electrophoresis, I found that the results (Figure 1) varied greatly due to the lack of standardization of the techniques. This led to my seeking an efficient method that would yield the most consistent results.

The first procedure that was examined was the homogenization-sonification step. It was found that the buffer

in which the ova were homogenized and sonicated needed to be of a high ionic strength (0.25 M NaCl) (Dejmal and Brookes, 1972) and also 1-2 pH units above the yolk proteins assumed isoelectric point. When a buffer of lower ionic strength and lower pH was used for homogenization and sonication, the vitellin was poorly extracted from the ova and poor yields were obtained (Figure 2). When the vitellin was properly extracted ~15 mg of vitellin was yielded per ovaries of 150-200 mosquitoes after chromatography (this was estimated on the basis of the 260 nm/280 nm ratio, see Materials and Methods). Extraction was considered less than acceptable if less than half this amount of vitellin was recovered. In the extraction procedure used to obtain the data in Figure 2B, approximately 1 mg of vitellin was recovered. It was found that a 0.05 M Tris-phosphate buffer with 0.25 M NaCl at pH 8 was the most efficient buffer for extracting the vitellin (Appendix B). Figure 2A shows vitellin prepared by homogenizing and sonicating in the pH 8, 0.05 M Tris-phosphate buffer containing 0.25 M NaCl and chromatographed. Figure 2B shows a sample eluted off the DEAE column which had been homogenized in phosphate buffered saline (PBS) (Appendix B), and Figure 2C shows the pre-salt rinse from the DEAE column run to elute the sample in Figure 2B, this illustrated that the problem was not a lack of protein binding to the column but rather that the protein

was not extracted from the ova. Figure 2D shows an unsuccessful attempt to examine the subunits of Figure 2B, by electrophoresis in 5% acrylamide gels containing 1% SDS. Hagedorn and Judson (1972) used a 0.05 M Tris Chloride buffer, (pH 8.5) with 0.5 M NaCl to extract vitellin from freshly laid eggs (instead of ova) which seemed to be an effective method since the buffer was of both high ionic strength and pH. The results of Oie et al., (1975) agree with my results. They demonstrated that a buffer of pH 8 with 0.4 M NaCl more efficiently and selectively extracted vitellin than any other solutions and buffers tested.

Another difficulty arose when I found a discrepancy in my results when using a Na-Na buffer pH 7 (Hagedorn and Judson, 1972) to solubilize and elute the vitellin off of the DEAE column (Appendix B). At times I was able to recover the vitellin from the column in greatly reduced quantities, compared with the amount I applied to the column, (the method for quantifying is previously described) and there was an additional, less distinct band appeared above the major vitellin band (Figure 3C). At other times I was unable to recover any of the vitellin that was applied to the DEAE column. It was also apparent that something came off the column when it was washed with 0.1 M NaCl. This could be due to the vitellin not completely binding to the column, or it could possibly be that vitellin is more than one protein, and one or more proteins of the complex is eluted at this molarity (Figure 3D).

When I changed to a Na-K buffer at pH 7 the pattern changed (Appendix B). The reason for the drastic difference in the reproducibility of the two different buffers is that the Na-Na buffer is a weaker buffer than the Na-K buffer. This may indicate that when the vitellin was in a high enough concentration in the Na-Na buffer it tended to have a "buffering" effect on the buffer, pulling the pH of the Na-Na buffer towards its assumed isoelectric point. This causes the vitellin to precipitate out of solution, thus preventing it from entering and binding to the DEAE column. On the other hand, the pH 7 Na-K buffer had stronger buffering capacities, so the vitellin may have tended to stay in solution at the higher concentrations and could be eluted from the DEAE column successfully. Hagedorn and Judson (1972) used a 0.1 M Na-Na phosphate buffer, pH 7 to solubilize and elute vitellin (eluting buffer contained 0.25 M NaCl) successfully. But, they never used solutions of high concentration (not greater than 5 mg/ml) and therefore they did not encounter the problem of the vitellin precipitating out of solution.

Figure 3 is a comparison of the unfed female-male extract (Figure 3A) with purified vitellin (Figure 3B) and samples from a run with the pH 7 Na-Na buffer (Figure 3C and (Figure 3D). The unfed female-male extract contained whole mosquitoes, with the preparative procedures being the same as for the ova cellular extract up to the point of

chromatography. Comparison of the unfed female-male extract with the purified vitellin indicates only that vitellin may be a blood-fed female specific protein which does not occur in unfed females or males since it is only a gross comparison.

After vitellin was eluted off the DEAE column, gel electrophoresis was performed on the vitellin to check its purity. [I used the Laemmli technique and variations of it (Laemmli, 1970; Schrader and O'Malley, 1977) because of the high capacity for salt and resolution of the protein bands]. During this process the necessity for recrystallizing acrylamide was discovered (Appendix C). It presently appears significant that it was recrystallized in ethyl acetate (Appendix C), because others have reported that acrylamide recrystallized in chloroform gave the same results as if the acrylamide had not been recrystallized (Kaczor, personal communication).

Figure 7 shows the well defined difference between gels containing recrystallized acrylamide (Figure 7A and Figure 7C) and gels containing unrecrystallized acrylamide (Figure 7B and Figure 7D). Figure 7E as compared to Figure 7A or 7C, illustrates the fact that precolumn vitellin run in gels containing recrystallized acrylamide had only a small amount of contamination. In contrast, precolumn vitellin run on gels with unrecrystallized acrylamide (Figure 7F) appears to contain more contamination. What exactly caused the

aberrant banding patterns in the gels containing the unrecrystallized acrylamide is unknown. It is possible that they were due to a protease in the unrecrystallized acrylamide breaking down the protein. However, because of the positions and consistency of the contaminating bands, it would seem more likely that they were due to contaminants in the acrylamide that are removed in the recrystallization process. The picture of vitellogenin, banding patterns presented by Oie et al., (1975) were similar to those of Figure 7B and 7D. This might indicate that they neglected to recrystallize their acrylamide or that they recrystallized it in chloroform. Still, it is difficult to make comparisons with other systems, since most investigators neglect to mention, (1) whether the acrylamide was recrystallized or unrecrystallized or (2) the solvent system used, if the acrylamide is recrystallized. An important piece of data that should again be noted in Figure 7 is that there is no appreciable difference between the chromatographed (Figure 7A and 7B) and the non-chromoatographed vitellin (Figure 7E). Since the precolumn preparation of the vitellin in Figure 7A through Figure 7F was identical, this would seem to indicate either (1) that a complex of soluble ova proteins is contained in the single band or that (2) that a single complex protein was isolated by the precolumn preparative procedures. A discussion of this has been previously presented in the General Introduction of this section.

The problem of unrecrystallized versus recrystallized acrylamide does not seem to be as great when running SDS acrylamide gels, as can be seen in Figure 8. There is little difference between recrystallized and unrecrystallized acrylamide, though the subunits on the recrystallized acrylamide tend to separate somewhat better and the resolution is clearer. (The dark staining, narrow band near the bottom of the gels illustrated in Figure 8 is probably due to contamination of the dye used to indicate the front).

Another important factor to be considered when examining the purity of vitellin on acrylamide gels is the concentration of protein in the sample applied to each gel. Figure 5 shows results of vitellin from the same sample applied to gels in increasing concentrations. In Figure 5A, where a low concentration of the protein was applied to the gel there was a "masking" of the contaminating band. In Figure 5C at a higher concentration, the contaminating band is clearly visible above the major vitellin band. This shows that the vitellin eluted off the DEAE with the Na-Na buffer contained impurity. A similar problem arose when running SDS acrylamide gels to examine the subunits of vitellin as is seen in Figure 6. When a concentration of 50 µg (Figure 6A) was applied to the gel the top band was resolved into 2 bands and the 2 bottom bands became more distinct. In Figure 6B where a 100 µg aliquot of vitellin was applied to

the gel, the 2 top bands became indistinguishable. If 100 µg was the only concentration of vitellin used, 5 major subunits would be postulated instead of the 6 that are actually present.

When separating the subunits of vitellin in the SDS acrylamide gels, I boiled the vitellin sample for 6 minutes before applying it to the gels to see if there was a need to break down any molecular interactions that might not allow the protein to completely separate into its subunits (Wyatt, Hagedorn personal communication). However, as can be seen in Figure 8, there is no difference between samples that were boiled before electrophoresing (Figure 8A and 8B) and samples which were not (Figure 8C and 8D). Hence, there is no indicating that heat should be applied to the vitellin to facilitate its separation into subunits in SDS acrylamide gels. This would seem reasonable since the sample had been previously dialyzed against the BME solution (Appendix D) which contains potent denaturing agents, so boiling would not be necessary since complete separation had already occurred.

As a matter of interest the efficiency of 2 reservoir buffers was examined. The gels illustrated in Figure 8 are 7.5% acrylamide gels, 1% SDS electrophoresed in Tris-glycine buffer (Appendix D). The gels shown in Figure 4D and Figure 4E were 5% acrylamide gels with 1% SDS also electrophoresed

in Tris-glycine buffer. Figure 4B and 4C show 7.5% acrylamide gels with 1% SDS electrophoresed in α-amino-n-butyric acid buffer (Appendix D). The only real differences between the two buffer systems was that the subunits in the gels electrophoresed in the α-amino-n-butyric acid system migrated proportionally farther down the gel and the electrophoretic process took 4-6 hours longer than those gels electrophoresed in the Tris-glycine buffer system. Since the differences were not major the Tris-glycine buffer system was routinely used to standardize my procedures.

To examine which subunits have carbohydrate moities attached a modification of the procedure presented by Zacharius et al., (1969) for staining acrylamide gels with periodic acid Schiff's (PAS) stain was used (Appendix E). I first attempted to follow their method precisely, but I was unable to remove the background stain from the gels, even after extensive rinsing with distilled water for 2 days. This effect was probably due to the SDS (which Zacharius et al., did not have in their gels) non-selectively binding the stain. To alleviate this problem the gels were rinsed overnight in distilled water after fixation in the trichloroacetic acid (TCA) and before the staining process. With other slight modifications in timing the procedure proved successful. The data in Figure 4E shows that all the major subunits stained positive for carbohydrates. This picture appears to show

that the staining was poor, but in fact this was not the case, rather it was due to the fact that the PAS stain is pink in color and the contrast between the stained bands and white background was poor in comparison with the deep blue color of the protein stain (Figure 4D) and the white background.

The last staining process was used to examine the lipid moieties attached to the subunits. The process for staining acrylamide gels for lipid moieties as proposed by Whittaker and West (1962) (Appendix E) was a complete failure with the system I used. The stain precipitated out during the staining procedure and produced blotchy grey-colored gels. A more successful method was the prestaining technique of Narayan et al., (1966) (Appendix E). This involved staining the vitellin sample before electrophoresis and produced gels with all the subunit bands stained, though very lightly. This light staining was probably due to the low concentration of the vitellin in the sample, rather than to the procedure itself. Another problem with this method was that the stain faded from the gels upon storage, thus making it necessary to photograph the gels almost immediately after electrophoresis.

Amino Acid Analysis

On completion of the amino acid analysis of *Aedes aegypti* vitellin (as described in Materials and Methods), I needed to choose an appropriate quantitative method to compare my analysis with the analysis of other insect vitellins. The SΔQ method compares protein X and Y,

$$S\Delta Q = \sum_{i=1}^{16} (X_i - Y_i)^2$$

where X_i and Y_i refer to the mole percent of amino acid i in proteins X and Y respectively. This method emphasizes those amino acids that differ the most between two proteins since it squares each difference. It is appropriate for searching for differences between vitellins. Using this means of comparison, the maximum difference which values obtained for the same protein in different laboratories could produce is 4 SΔQ units. The theoretical maximum value among unrelated proteins is 20,000 SΔQ units which would be obtained in the comparison of two distinct homopolymers (Marchalonis and Weltman, 1971).

Values for cysteine and tryptophan were omitted because data for these proteins is often unreliable and these amino acids constitute only a small fraction of most proteins. Aspartate was combined with asparagine and glutamate was combined with glutamine since in most analysis they are not separated (Marchalonis and Weltman, 1971).

As can be seen from the data (Tables 1 and 2), most insect vitellins compared have SΔQ values that fall within the range of 21 to 77 SΔQ units. Given the upper limit of 20,000 SΔQ units for two completely unrelated proteins, this observation points to the fact that these proteins are closely related proteins. The one aberrant analysis on D. virilis vitellin (Kambysellis, 1977) is probably due to contamination, as is indicated by the abnormally high glycine content, rather than the unrelatedness of the vitellin.

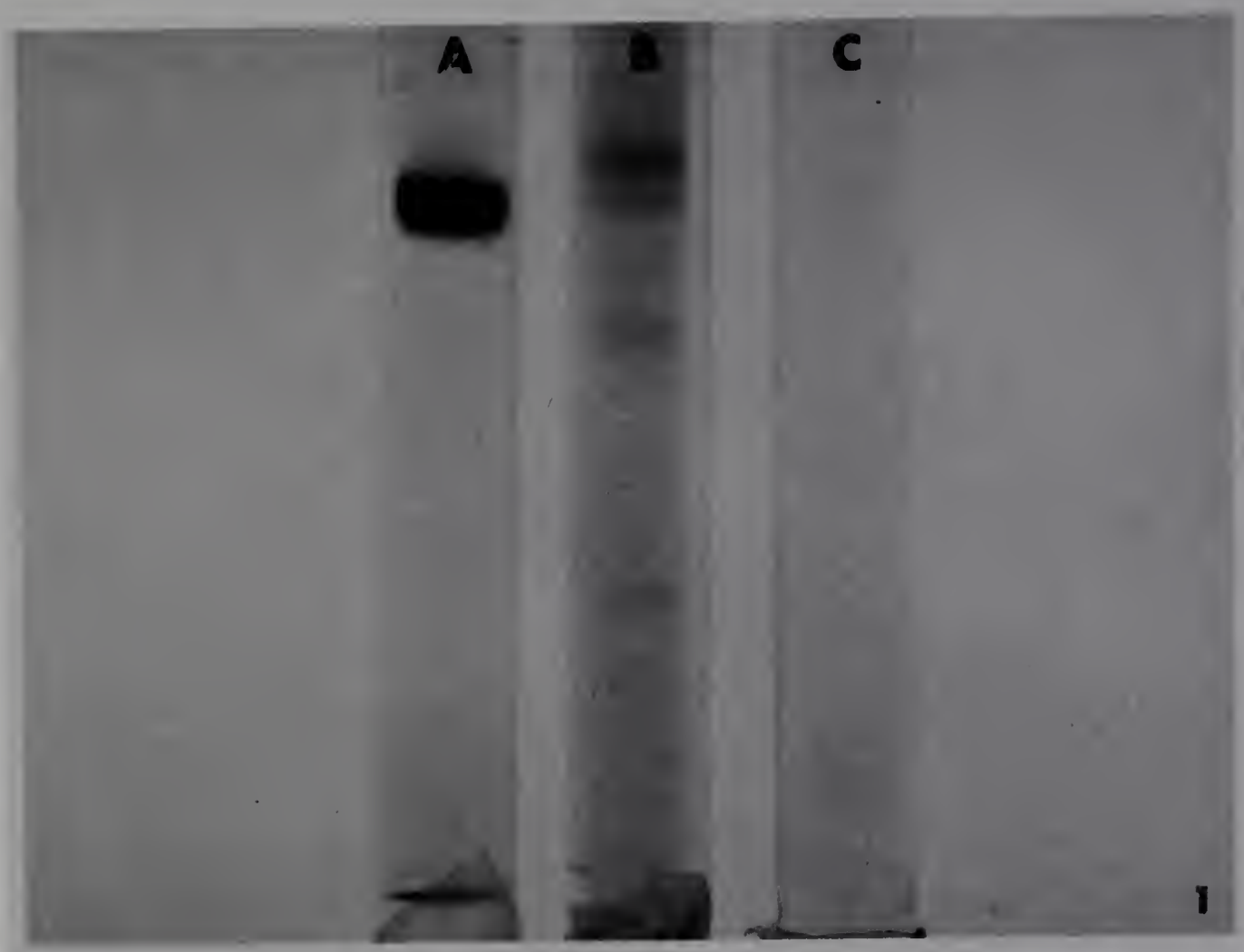
It is also interesting to note that the SΔQ values for the vitellin-nonvitellin comparisons are somewhat higher than the SΔQ values for the vitellin-vitellin comparisons (omitting D. virilis) (Table 2). The average SΔQ value for the vitellin-nonvitellin comparisons was 80 SΔQ units, as compared to the average SΔQ value of 47 for the vitellin-vitellin comparisons. Given the range of SΔQ values of 21 to 77 SΔQ unit when comparing vitellins (omitting D. virilis), this difference would appear somewhat significant and indicate that vitellins are more related to each other than to other insect proteins.

It has been suggested that vitellins are a rapidly evolving group of proteins since the only restrictions on them as a group are that (1) they must remain large enough to avoid being filtered by the pericardial cells, (2) they must retain a recognition site for uptake into the oocytes and (3) they must maintain a "nutritive" amino acid

composition (Kunkel and Pan, 1976). King and Jukes (1969) have predicted a mutational equilibrial amino acid composition on the assumption that vitellins are a rapidly evolving group. Such an equilibrium would give all typical vitellins a similar amino acid composition, even if they diverged from each other very rapidly in absolute sequence (King and Jukes, 1969). Of the 9 amino acid analysis of insect vitellins including my analysis on A. aegypti vitellin, all but one have compositions that fall close to the "observed average protein" (OAP) (Table 3). The D. virilis vitellin is again the one exception and should be discounted because of the uncertainty of the quality of the analysis. Viewing these observations it is clear that vitellins do tend to cluster about an amino acid composition that is close to the OAP composition. And since the methods now available for doing amino acid analysis are not sensitive enough to detect small differences in amino acid composition in such large proteins as vitellins, we cannot expect to gain much useful information from future comparisons of such analysis from different insect vitellins. Rather, it might be more profitable to concentrate on peptide mapping and sequencing to compare vitellins in the future (Kunkel and Hagedorn, unpublished).

Figure 1. (All gels are 6% acrylamide gels with recrystallized acrylamide). (A) purified vitellin; (B) vitellin eluted off a DEAE column using a Na-K buffer, pH 6.8; (C) vitellin that was eluted off a DEAE column with a Na-Na buffer (in this case the vitellin precipitated out of solution on top of the DEAE column and could not be recovered).

Figure 2. (A, B, and C are 6% acrylamide gels with recrystallized acrylamide, D is a 5% acrylamide gel, 1% SDS with recrystallized acrylamide). (A) purified vitellin; (B) vitellin that was homogenized in PBS and eluted off a DEAE column; (C) presalt wash of the preparative procedure for (B) before 0.25 M NaCl solution was applied to elute the vitellin; (D) "subunits" of (B).


A
B
C
1

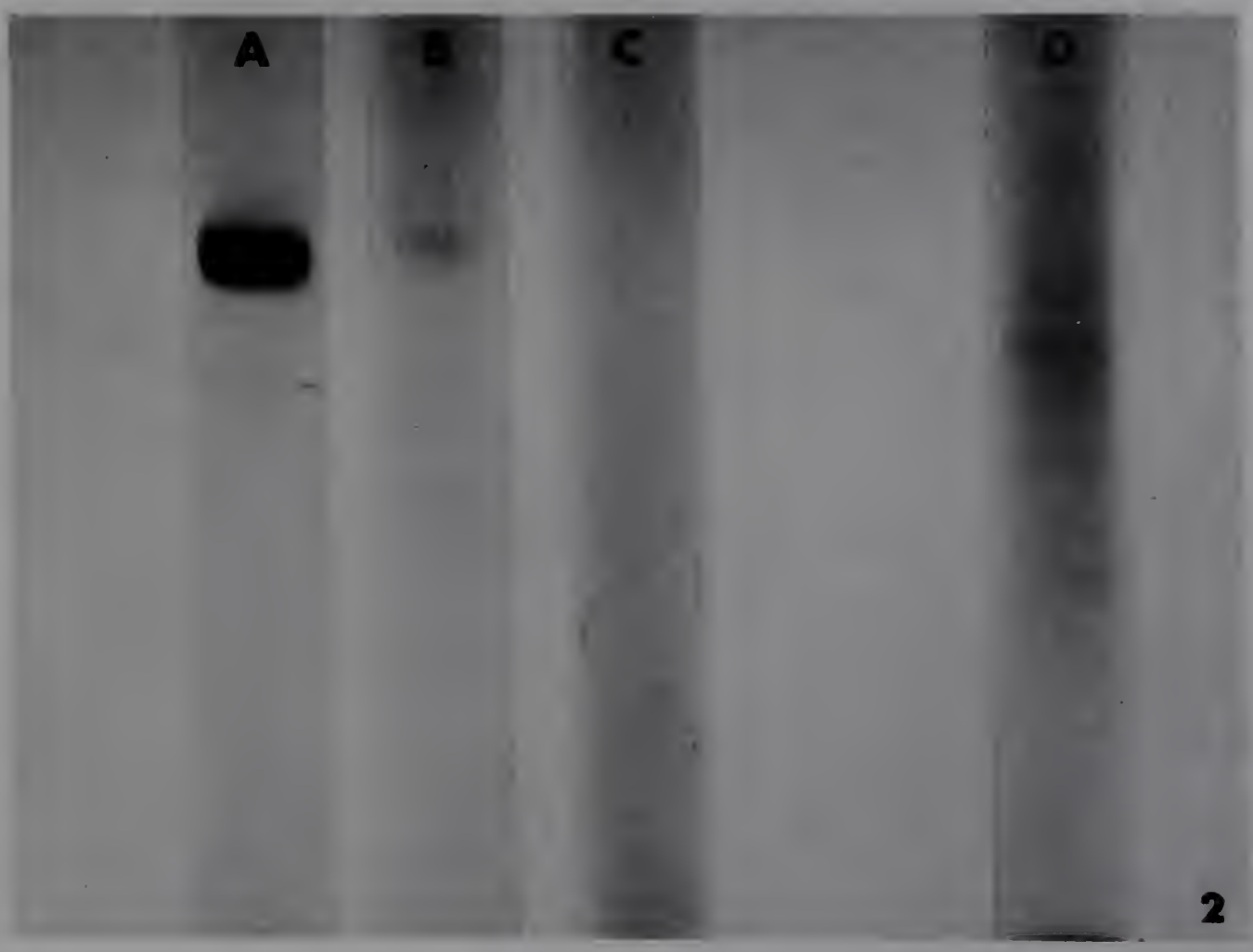

A
B
C
D
2

Figure 3. (All gels are 6% acrylamide gels with recrystallized acrylamide). (A) male-unfed female extract; (B) purified vitellin, (C) vitellin eluted off the DEAE column with a Na-Na buffer (in this case the vitellin was applied to the column and only a small fraction was recovered), (D) vitellin eluted off a DEAE column with a 0.1 M NaCl Na-Na buffer (vitellin is normally eluted off the column in 0.25 M NaCl buffer).

Figure 4. (A) purified vitellin on 6% acrylamide gels with recrystallized acrylamide; (B) subunits of (A) 50 µg of vitellin run on 7.5% acrylamide gels, 1% SDS using the α-amino-n-butyric acid system; (C) subunits of (A) -25 µg of vitellin run on 7.5% acrylamide gels, 1% SDS using the α-amino-n-butyric acid system; (D) subunits of (A) vitellin run on 5% acrylamide gels, 1% SDS using the Tris glycine system; (E) same as (D), except PAS stain was used instead of Coomassie Brilliant Blue.

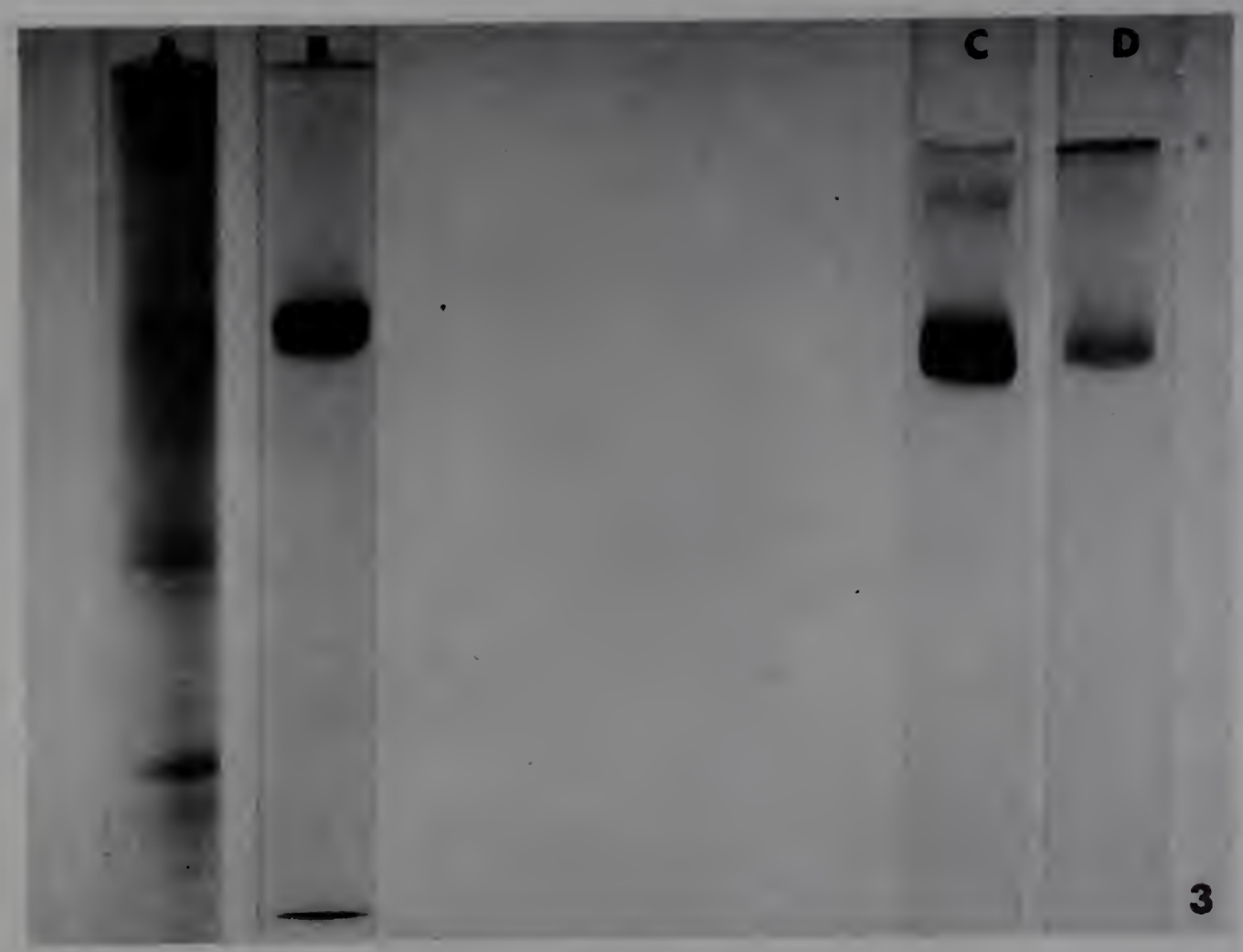
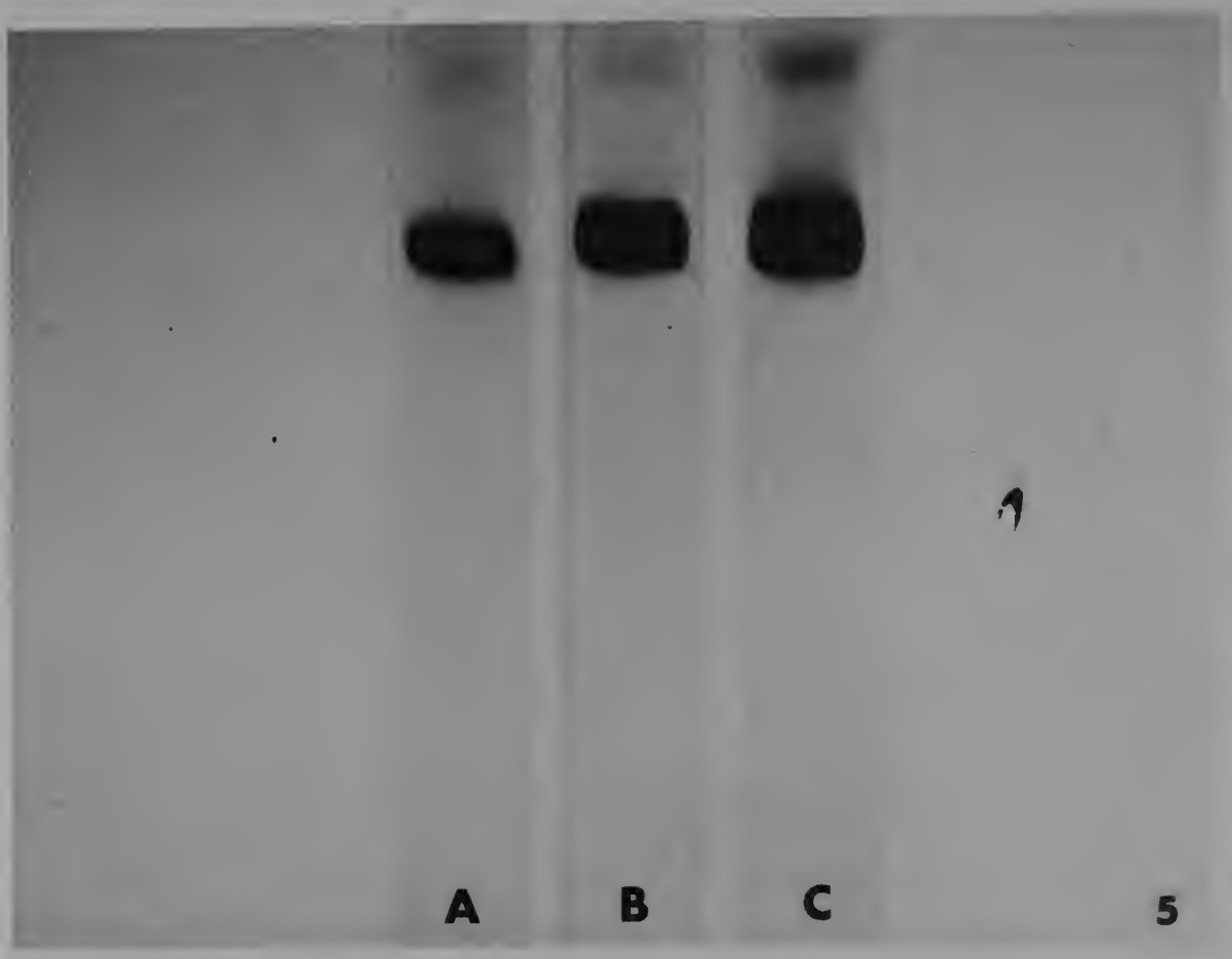
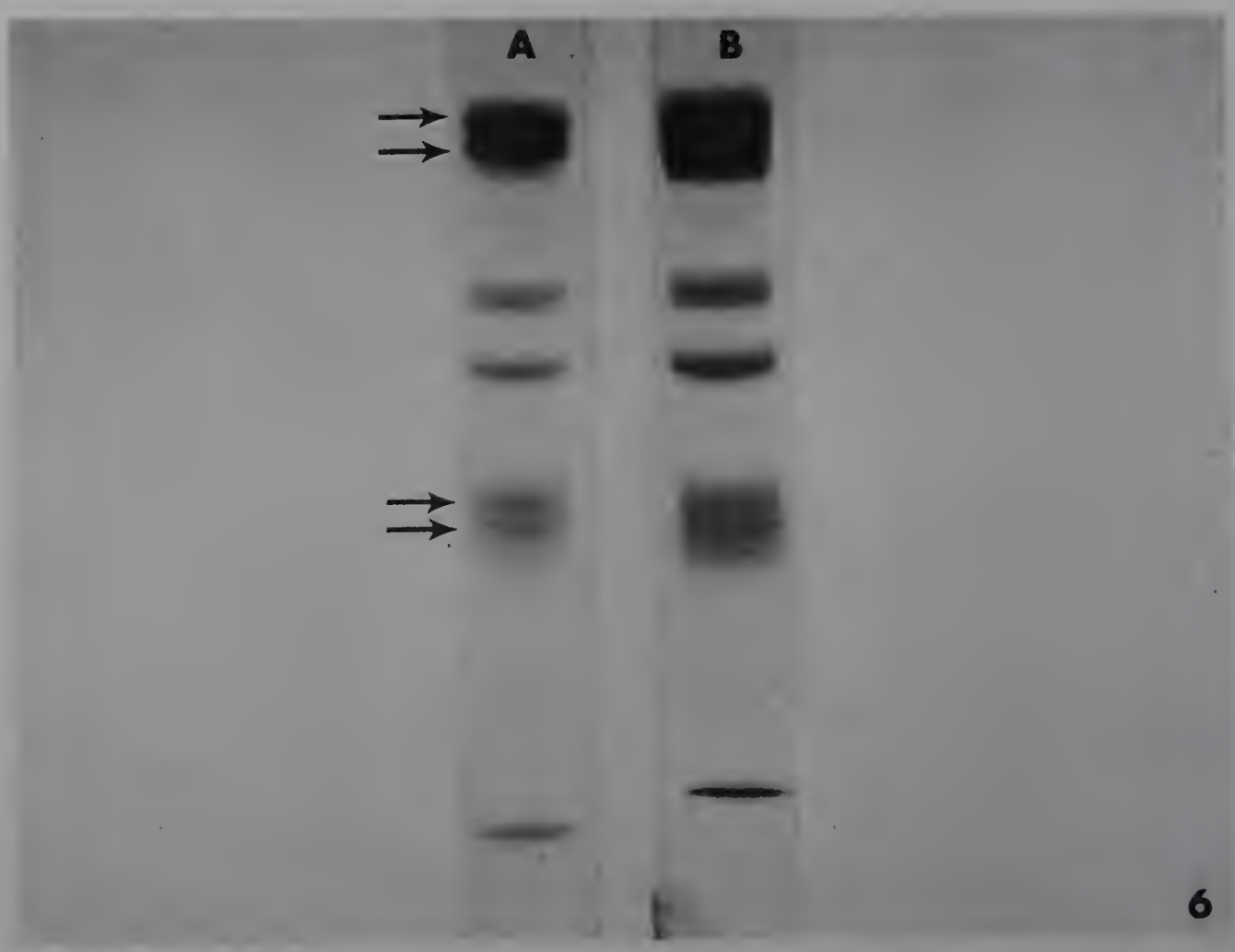

C
D
3

Figure 5. Vitellin samples were eluted off a DEAE column using a Na-Na buffer. All samples were run on 6% acrylamide gels containing recrystallized acrylamide. This shows the effects of masking due to the concentration of the samples run on the gels. (A) 40 µg, (B) 60 µg, (C) 80 µg (aberrant band above the major vitellin band is now clearly visible).

Figure 6. Vitellin samples were run on 5% acrylamide, 1% SDS gels, (A) 50 µg of vitellin, 2 top bands are distinguishable; (B) 100 µg of vitellin, 2 top bands are indistinguishable.



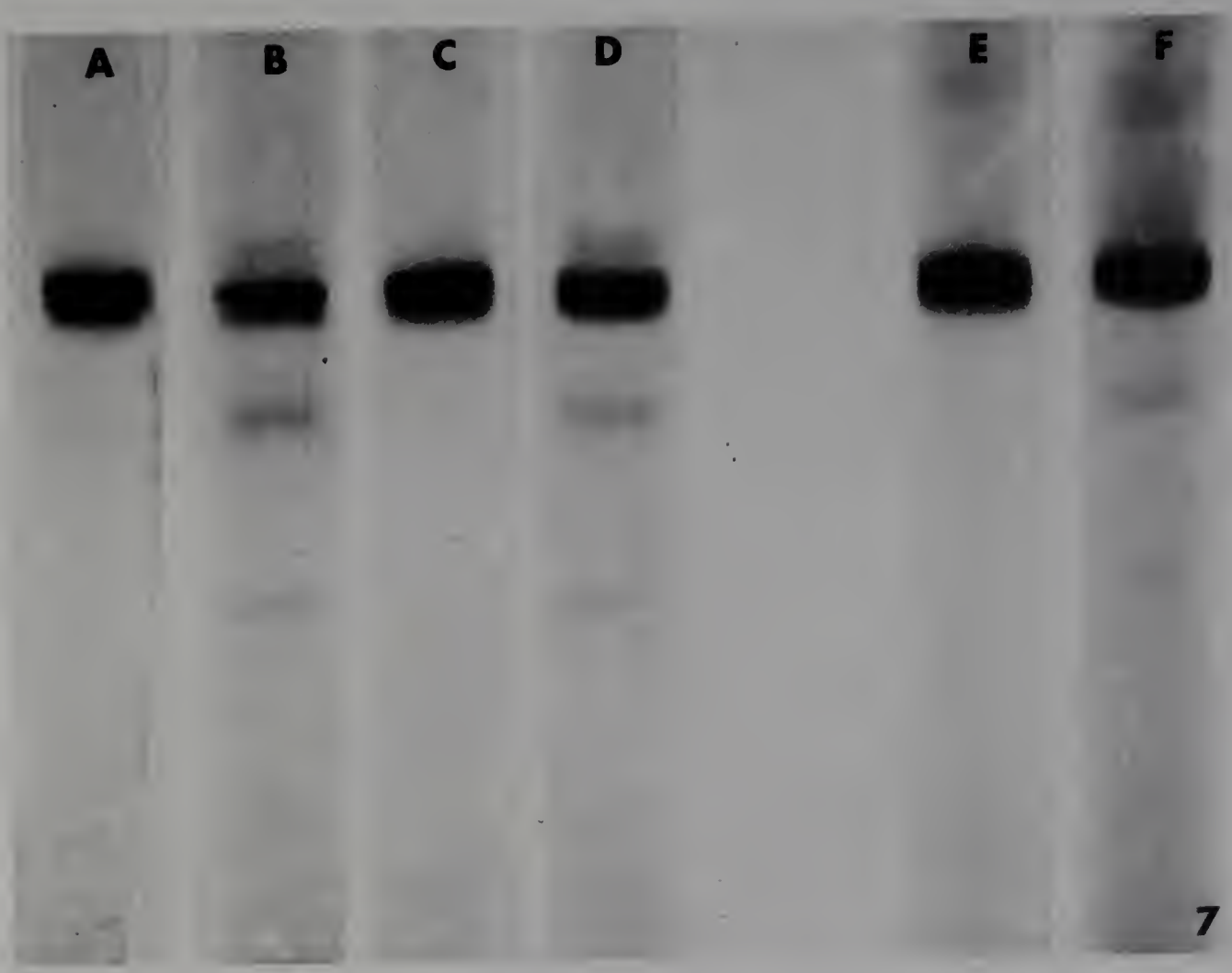
5



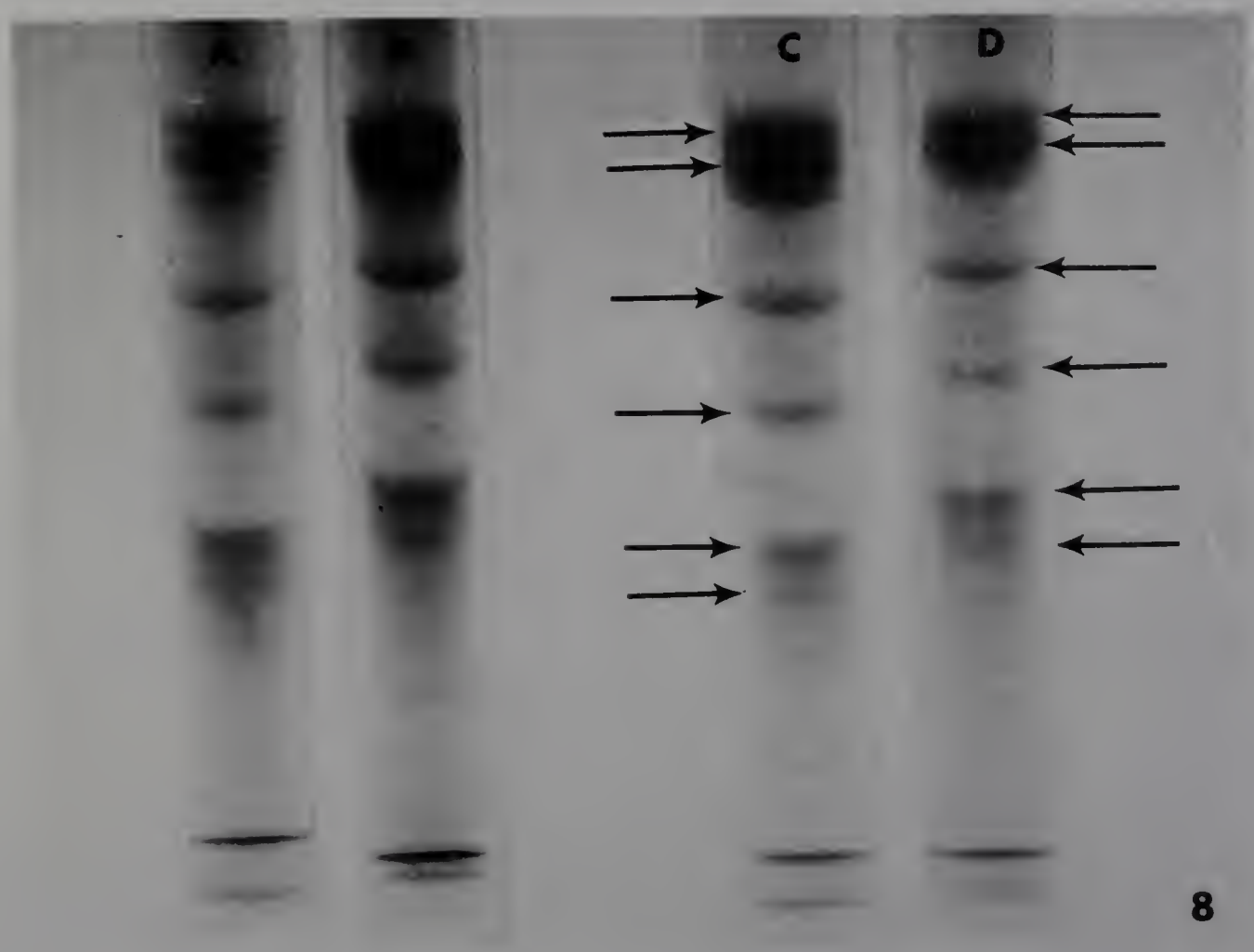
6

Figure 7. Vitellin samples were eluted off a DEAE column in a Na-K buffer, pH 7 with 0.25 M NaCl. All gels are 6% acrylamide gels. (A) and (C) are purified vitellin run on recrystallized acrylamide, (B) and (D) are purified vitellin run unrecrystallized acrylamide, (E) precolumn vitellin run on recrystallized acrylamide, (F) precolumn vitellin run on unrecrystallized acrylamide.

Figure 8. Vitellin samples prepared the same as those that were chromatographed in Figure 7. All gels are 7.5% acrylamide gels, 1% SDS. (A) Vitellin sample that was dialyzed against BME solution ~12 hr and not boiled before applying it to the gel containing recrystallized acrylamide; (D) vitellin was prepared the same as for (C), but the gel contained unrecrystallized acrylamide.



7



8

TABLE 1

MOLE PERCENT AMINO ACID COMPOSITION OF VARIOUS INSECT VITELLINS AND NON-VITELLINS

	A. aegypti (my data)	L. migratoria (Gellissen)	B. germanica (Kunkel and Pan)	H. cercropia (Kunkel and Pan)	P. cynthia (Chino et al., 1977b)	L. decemlineata (Deloof and DeWilde)	D. melanogaster (Gingeras)	D. virilis (Kambysellis)	L. madarae (Engleman and Friedal Dejmal and Brookes) (OAP)	Observed average protein (as suggested by King and Jukes)	B. germanica serum protein I (Kunkel and Pan)	B. germanica serum protein II (Kunkel and Pan)
Asx	12.7	10.8	12.2	9.7	10.2	10.0	11.4	8.1	15.0	10.8	12.0	10.9
Thr	4.3	4.9	5.7	5.1	5.2	5.4	5.8	4.7	5.4	6.5	3.4	6.1
Ser	8.8	8.2	9.1	9.2	7.7	9.7	6.2	13.5	7.6	8.5	3.5	6.3
Glx	11.9	12.8	10.9	14.3	15.7	12.8	10.1	11.9	11.2	10.0	11.7	11.2
Pro	5.3	7.0	4.3	4.4	5.4	5.2	5.2	2.6	4.8	5.2	5.8	3.62
Gly	4.4	5.1	3.0	4.4	4.6	5.2	9.2	31.3	3.3	7.8	4.4	6.7
Ala	6.3	8.2	4.9	7.5	7.2	5.5	8.5	7.5	5.4	7.8	5.1	7.0
Val	6.6	7.2	8.5	5.6	6.0	6.8	6.4	3.7	6.8	7.1	7.7	9.2
Met	1.1	1.5	2.8	2.0	2.1	2.5	1.9	0.5	2.0	1.9	2.6	0
Ile	4.7	4.5	5.2	4.5	4.7	5.7	5.0	2.1	4.9	4.0	3.8	2.4

TABLE 1--Continued

	A. aegypti (my data)	L. migratoria (Gellissen)	B. germanica (Kunkel and Pan)	H. cercropia (Kunkel and Pan)	P. cynthia (Chino et al., 1977b)	L. decemlineata (Deloof and DeWilde)	D. melanogaster (Gingeras)	D. virilis (Kambysellis)	L. madarae (Engleman and Friedal Dejmal and Brookes) (OAP)	Observed average protein (as suggested by King and Jukes)	B. germanica serum protein I (Kunkel and Pan)	B. germanica serum protein II (Kunkel and Pan)
Leu	6.1	9.8	8.6	6.0	6.5	7.0	8.0	4.9	9.2	8.0	6.1	10.4
Tyr	5.0	5.8	4.4	5.2	4.8	3.8	3.9	2.0	4.5	3.5	8.9	3.0
Phe	5.9	2.8	4.4	3.5	3.2	4.7	2.8	1.7	4.2	4.2	6.4	5.0
Lys	9	5.6	7.3	7.5	8.9	7.8	7.5	3.0	6.1	7.5	8.0	9.2
His	4.4	1.8	4.3	3.1	3.4	2.9	2.2	0.4	3.2	3.0	4.9	4.5
Arg	3.4	4.0	4.3	7.9	4.2	5.0	5.9	2.2	6.5	4.4	4.7	3.4

Asx = aspartate and asparagine
Thr = threonine
Ser = serine
Glx = glutamate and glutamine
Pro = proline
Gly = glycine
Ala = alanine
Arg = arginine
Val = valine
Met = methionine
Ile = isoleucine
Leu = leucine
Tyr = tyrosine
Lys = lysine
His = histidine

TABLE 2

COMPARISON OF MOLE PERCENT AMINO ACID COMPOSITIONS FROM VARIOUS INSECT VITELLINS AND NON-VITELLINS USING SΔQ ANALYSIS (KUNKEL AND HAGEDORN, 1978 UNPUBLISHED, KING AND JUKES, 1969, MARCHALONIS AND WELTMAN, 1971)

	A. aegypti	L. migratoria	L. madarae	B. germanica	H. cercropia	P. cynthia	L. decemlineata	D. melanogaster	D. virilis	OAP	B. germanica serum protein I	B. germanica serum protein II
A. aegypti		56	44	28	50	34	24	76	868	41	54	60
L. migratoria			49	48	51	40	38	47	812	37	93	68
L. madarae				23	63	71	47	68	962	56	86	122
B. germanica					59	54	23	77	951	42	77	60
H. cercropia						21	24	67	843	57	96	100
P. cynthia							23	67	847	54	79	75
L. decemlineata								54	795	28	85	64
D. melanogaster									635	15	104	55
D. virilis										670	995	798
OAP											101	34
B. germanica serum protein I												105
B. germanica serum protein II												

SUMMARY AND CONCLUSIONS

General Introduction

From an examination of my data, and a survey of the limited, existing information about the biochemical nature of vitellin, I cannot assume that vitellin is a single protein. Rather, I must consider two options, (1) that vitellin is a complex mixture of very related, soluble proteins, predominantly made up of yolk proteins (Telfer, 1960), or (2) that vitellin is a single very complex protein. Further, more thorough research must be done on vitellin before any conclusions on this matter can be reached.

Isolation, Purification and Gel Electrophoresis of the Vitellin

After surveying the various techniques of isolation, purification and gel electrophoresis of vitellin and examining the methods I used, it became obvious that there was need for standard, well-defined techniques to be developed. Therefore, the first objective of this research was to put forth techniques that gave reliable, consistent results when isolating, purifying and performing gel electrophoresis on A. aegypti vitellin. The available data and the evidence gathered in this research support the following conclusions:

(1) The pH and ionic strength of the homogenizing-sonicating buffer is important to the isolation and later purification of the vitellin. The buffer must be of high ionic strength and usually of a pH 1-2 units above the isoelectric point of the yolk protein. For A. aegypti vitellin a 0.05 M Tris-Phosphate buffer with 0.25 M NaCl at a pH of 8.0 proved to be the most efficient for this purpose.

(2) When dealing with high concentrations of vitellin (greater than 5 mg/ml) it is important to use a strong buffer to solubilize the vitellin and elute it off the DEAE column. The pH of the eluting buffer is very important and must be maintained in order to purify the vitellin off of the DEAE column. A 0.1 M Na-K phosphate buffer at pH 7 with 0.25 M NaCl was found to be the most effective buffer for this purpose.

(3) When performing gel electrophoresis it is of extreme importance that the acrylamide be recrystallized in ethyl acetate before being used to make up the gel solutions. Unrecrystallized acrylamide in the gels causes the presence of aberrant bands. Moreover, it is also important that the electrophoresing times, amperage and temperatures be consistent throughout all the experiments. Variations in these parameters makes it extremely difficult to make any accurate comparisons between experiments.

(4) It is important that more than a single concentration of vitellin be examined when performing gel

electrophoresis. "Too much" or "too little" sample can cause "masking" of what is actually present in the sample.

(5) There is no need to heat the vitellin sample before applying it to SDS acrylamide gels to facilitate its separation into subunits, as would be expected since the sample had been previously treated with a strong denaturant.

(6) All of the subunits of the vitellin stain positive for carbohydrate using a PAS stain.

(7) The pre-staining technique (Narayan et al., 1966) for lipid moieties in the subunits of the vitellin proved more useful than the lipid staining technique of Whittaker and West (1962) but there are still needs for improvement before any conclusions can be made from the results.

Amino Acid Analysis

Since no amino acid analysis from a nematocerous Diptera had been carried out, the second objective of this research was to prepare and carry out an amino acid analysis of A. aegypti vitellin and make a comparison with the analysis of other insects. The available data and the evidence gathered from this analysis support the following conclusions.

(1) A. aegypti vitellin is closely related in amino acid composition to the other insect vitellins examined.

(2) Due to the insensitivity of the methods now available for doing amino acid analysis, and the inability to detect small differences in proteins as large as vitellins, it would appear that the usefulness of continuing in this line of making comparisons is very limited.

REFERENCES CITED

Brookes, V. J., Dejmal, R. K. "Yolk protein: structural changes during vitellogenesis in the cockroach Leucophaea madarae" 1968 Science 160:999-1001.

Chapman, J. M., Mills, G., Ledford, J. H. "The distribution and partial characterization of the serum apoliproteins in the guinea pig" 1975 Biochem. J. 149:423-436.

Chen, T. T., Couble, P. DeLucca, F. L., Wyatt, G. R. 1976 In The Juvenile Hormones, ed L. J. Gilbert, pp. 505-529. New York: Plenum Press, 572 pp.

Chino, H., Yamagata, M., Takahashi, K. "Isolation and characterization of insect vitellogenin. Its identity with hemolymph lipoprotein II." 1976 Biochim Biophys. Acta 441:349-353.

Chino, H., Downer, R. G. H., Takahashi, K. "The role of diacyl glycerol-carrying lipoproteins I in lipid transport during insect vitellogenesis" 1977a Biochim Biophys Acta 487:508-516.

Chino, H., Yamagata, M., Sato, S. "Further characterization of lepidopteran vitellogenin from haemolymph and mature eggs. 1977b Insect Biochem 7:125-131.

Dejmal, R. K., Brookes, V. J. "Insect Lipovitellin" 1972 J. Biol. Chem 247:869-874.

Deloof, A., DeWilde, J. "The relationship between Haemolymph proteins and vitellogenesis of the Colorado Potato Beetle Leptinotarsus decemlineata" 1970 J. Insect Phys. 16:157-169.

Engelmann, F., Friedel, T. "Insect yolk protein precursors, A juvenile hormone induced phosphoprotein" 1974 Life Sciences 14:587-594.

Engelmann, F., Friedel, T., Ladduwahetty, M. "The native vitellogenin of the cockroach, Leucophaea madarae" 1976 Insect Biochem 6(3):211-220.

Gellisen, G., Wajc, E., Cohen, E., Emmerich, H., Applebaum, S., Fossodorf, S. "Purification and properties of ooycte vitellin from the migratory locust 1976 J. Comp. Physiol. B. 108(3):287-301

Gingeras, T. H., Gelti-Douka, H., Kambysellis, M. P. "Yolk proteins in Drosophila. 1973 Dros Inf Serv 50:58.

Gingeras, T. H. "Identification, isolation and characterization of the yolk protein from D. virilis and D. melanogaster" 1976 Ph.D. Diss. New York University.

Hagedorn, H. H., Judson, C. L. "Purification and site of synthesis of Aedes aegypti yolk protein" 1972 Exp Zool. 182:367-377.

Hagedorn, H. H. Fallon, A., Laufer, H. "Vitellogenin synthesis by the fat body of the mosquito Aedes aegypti: evidence for transcriptional control" Dev Biol 31(2) 283-294.

Hagedorn, H. H. "The control of vitellogenesis in the mosquito, Aedes aegypti" 1974 Amer Zool 14:1207-1207.

Hagedorn, H. H., Turner, S., Hagedorn, E. A., Pontecorvo, D., Greenbaum, P., Pfeiffer, D., Wheelock, G., Flanagan, T. R. "Postemergence growth of the ovarian follicles of Aedes aegypti" 1977 J. Insect Physiol 23:203-206.

Hagedorn, H. H., Kunkel, J. G., Wheelock, G. "The specificity of an antiserum against mosquito vitellogenen and its use in a radio immunological precipitation assay for protein synthesis" 1978 In press.

Kambysellis, M. P. "Genetic and Hormonal Regulation of Vitellogenesis in Drosophila. 1977 Amer Zool 17:535-549.

Kane, J. P. "A rapid electrophoretic technique for identification of subunits species of apoproteins in serum lipoproteins" 1973 Anal Biochem 53:350-364.

Kelly, T. J., Telfer, W. H. "Antigenic and electrophoretic variants of vitellogenin in Oncopeltus blood and their control by juvenile hormone" 1977 Dev Biol 61:58-69.

King, T. H., Jukes, J. L. "Non-Darwian Evolution" 1969 Science Wash 164:788-798.

Kunkel, J. G., Hagedorn, H. H. 1978 Unpublished manuscript. Ann. Rev Ent.

Kunkel, J. G., Pan, M. L. "Selectivity of yolk uptake: comparison of vitellogenins of two insects." 1976 J. Insect Physiol. 22:809-818.

Laemmli, V. K. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4" 1970 Nature 227-080-685.

Laufer, H. 1964. In Taxonomic Biochemistry and Serology, pp. 171-189. ed. Leine, C. A. New York: Ronald Press, 728 pp.

Marchalonis, J. J., Weltman, J. K. "Relatedness among proteins: a new method of estimation and its application to immunoglobulins" 1970 Comp. Biochem Physiol 38B: 609-625.

McDonald, H. J., Ribeiro, L. P. "Ethylene and propylene glycol in the prestaining of lipoproteins for electropernesis" 1959 Clin Chem Acta 14(3)458-459.

McGregor, D. A., Loughton, B. G. "Yolk protein degradation during embryogenesis of the African migratory locust" 1974 Can J. Zool 52:907-917.

McGregor, D. A., Loughton, B. G. "Amino acid composition, degradation and utilization of locust vitellogenin during embryo genesis. 1977 Wilhem Roux Arch 181:113-122.

McGucken, W. F., McKenzie, B. F. "An Improved Perodic Acid Fuchsin Sulfate Staining Method for Evaluation of Glycoproteins" 1958 Clin Chem 4(6):476-483.

Narayan, K. A., Creinin, H. L., Kummerrow, F. A. "Disc electrophoresis of rat plasma lipoproteins" 1966 J. of Lipid Res 7:150-151.

Oie, M., Takahashi, S., Ishizaki, H. "Vitellogenin in the eggs of the cockroach Blatella germanica: purification and characterization" 1975 Dev. Growth Differ 17(3):237-246.

Orstein, L., Davis, B. J. In Disc Electrophoresis, reprinted by Distillation Products Industries, Rochester, New York, 1962.

Pan, M. L., Bell, W. J., Telfer, W. H. "Vitellogenic blood protein synthesis by insect fat body" 1969 Science 165:393-394.

Pan, M. L., Wallace, R. A. "Cercropia vitellogenin: isolation and characterization" 1974 Amer Zool 14:1239-1242.

Roth, T. E., Porter, K. R. "Yolk protein uptake in the oocyte of the mosquito Aedes aegypti 1964. J. Cell Biol. 20:313-330.

Schrader, W. T., O'Malley, B. In Laboratory Methods Manual for Hormone Action and Molecular Endocrinology Baylor College of Medicine, Houston.

Telfer, W. H. "Immunological studies of insect metamorphosis II The role of a sex-limited blood protein in egg formation by the Cercropia silkworm." 1954 J. Gen Physiol 37:539-558.

Telfer, W. H. "The selective accumulation of blood proteins by the oocytes of Saturniid moths" 1960 J. Biophys Biochem Cytol. 9:747-759.

Weber, K., Osborn, M. "The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis 1969 J. Biol Chem 244:4406-4412.

Whitmore, E., Gilbert, L. I. "Haemolymph proteins and lipoproteins in lepidoptera: a comparative electrophoretic study" 1974 Comp Biochem Physiol 47B:63-78.

Whittaker, J. R., West, A. S. "Starch gel electrophoretic study of insect haemolymph proteins" 1962 Can J. Zool 40:655-658.

Wyatt, G. R., Pan, M. L. "Insect Plasma Proteins" 1978 Ann Rev Biochem In press.

Zacharius, R. M., Zell, T. E., Morrison, J. H., Woodlock, J. J. "Glycoprotein staining from electrophoresis in acrylamide gels" 1969 Anal Biochem 30:148-152.

APPENDIX A

Aedes Saline (Hagedorn et al., 1977)

	mM
$MgCl_2.6H_2O$	0.6
KCl	4.0
$NaHCO_3$	1.8
NaCl	150.0
HEPES	25.0
$CaCl_2.2H_2O$*	1.7

*Calcium is added after pH is adjusted to 7.0 with NaOH.

APPENDIX B

(All buffers in this Appendix contain a 1:20 dilution of 6 mg/ml of PMSF in 95% ethanol and a 1:100 dilution of 0.2% sodium azide to prevent breakdown of vitellin by proteases and to prevent bacterial growth in the buffers respectively).

Homogenizing-sonicating Buffers

(1) 0.05 M Tris-Phosphate buffer, 0.25 M NaCl-pH 8.0. 0.05M Trizma Base, (Sigma) and 0.05 M NaH_2PO_4 mixed with 0.25 M NaCl and pH adjusted with NaOH (modified from Hagedorn et al., 1978).

(2) PBS (Phosphate Buffered Saline) made at a concentration of 10x. 1M KH_2PO_4, 0.9% NaCl pH adjusted with NaOH to 7.0 (Hagedorn personal communication). This buffer was used at a concentration of 1x.

Dialyzing and Eluting Buffers

(3) 0.05 M Tris-Phosphate buffer pH 6 - no NaCl. Made the same as (1), omit salt, pH adjusted to 6 with HCl.

(4) 0.1 M Na-K phosphate buffer pH 7. 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4 are mixed and pH adjusted to 7 with KOH at room temperature, (Hagedorn, personal communication). The buffer

is filtered through a Millipore filter then cooled to 4° before use. It is suggested that this buffer be made up fresh every 3-4 days.

(5) 0.1 M Na-K phosphate buffer, 0.25 M NaCl, pH 7- made the same as (4) with the addition of 0.25 M NaCl.

(6) 0.1 M Na-Na phosphate buffer pH 7. 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 are combined and pH adjusted with NaOH. Preparative procedure same as for (4).

(7) 0.1 M Na-Na Phosphate buffer pH 7, 0.25 M NaCl- made the same as (6) with the addition of 0.25 M NaCl.

APPENDIX C

Recrystallization of Acrylamide

(1) To recrystallize acrylamide dissolve acrylamide in boiling ethyl acetate, 400g/liter. Filter boiling solution through preheated Buchner funnel and vacuum flask with Whatman #1 filter paper. Let solution cool, then chill to $4^{o}C$, collect crystals and allow them to dry.

(2) To recrystallize methylene bisacrylamide follow above procedure using acetone and 10g/400ml (Hagedorn and Kaczor personal communication).

APPENDIX D

Reagents and Preparation of Gels (Modified from Laemmli, 1970)

30% acrylamide, 0.8% methylene bisacrylamide: 30 g acrylamide and 0.8 g methylene bisacrylamide are dissolved and adjusted to 100 ml with distilled water.

Lower Tris (4x): 18.17 Tris base, HCl to pH 8.8. Make up to 100 ml with distilled water (reagent should contain 4% SDS when preparing acrylamide gels with SDS).

Upper Tris (4x): 6.06 g Tris base, HCl to pH 6.8. Make up to 100 ml with distilled water (reagent should contain 4% SDS when preparing acrylamide gels with SDS).

Ammonium persulfate solution (Make up fresh each day) 100 mg ammonium persulfate and 5 ml of distilled water = 2% solution.

Tris-glycine buffer (4x):12 g Tris base and 57.6 g glycine. Make up to 1000 ml with distilled water.

α-amino-n-butyric acid buffer (4x) (Chapman et al., 1975):18.97 g α-amino-n-butyric acid made up to 1000 ml with distilled water.

BME solution ÷ 5 ml 2-mercaptoethanol, 30 ml 10% SDS and 12.5 ml upper tris (4x). Make up to 100 ml with distilled water.

Reservoir buffer: 500 ml of either Tris glycine buffer (4x) in α-amino-n-butyric acid buffer (4x), and distilled water up to 2000 ml (20 ml of 10% SDS should be added before bringing buffer up to 2000 ml when acrylamide gels with SDS are being electrophoresed).

Preparation of Gels

Lower gels for 12 gels, 12 cm long.
(in mls)

	5%	6%	7.5%
H_2O	22.7	20.3	19.35
Lower Tris (4x)	10.0	10.0	10.0
30% Acryl + 0.8% MBA	6.7	8.2	10.0
2% AP	0.6	0.6	0.6
Tetramethyethylenediamine (TEMED)	20 µl	20 µl	10 µl

Upper gels (for 12 gels)
(in mls)

H_2O

Upper Tris (4x)

30% acryl. + 0.8% MBA

2% AP

TEMED

APPENDIX E

Preparation of Stains and Staining Techniques

Coomasie Brilliant blue stain (modified from Laemmli, 1970). After electrophoresis, gels are fixed in Destain I (50% methanol, 9.5% glacial acetic acid) for 3-4 hrs. They are subsequently stained in a 1% solution of Coomasie Brilliant blue made up in Destain I. After staining overnight they are destained for 10-12 hrs in Destain I with 3 changes and then in Destain II (5% methanol 7.5% glacial acetic acid) for 12 hrs with 2 changes. Gels are stored in Destain II.

PAS stain (modified from Zacharius et al., 1969).

Reagents.

(1) 12.5% Trichloracetic acid made in distilled water.

(2) 1% Periodic acid in 3% acetic acid.

(3) Fushin-sulfite stain--10 gm of potassium meta-bisulfite and 21.0 ml of concentrated HCl are added to 7 liters of distilled water and mixed thoroughly, when a solution has been obtained 8 gm of basic Fushin stain is added and solution is stirred for 2 hrs at room temperature. The color should be a faint pink. Let solution stand for 2 hours then add an appropriate amount of decolorizing charcoal; stir and filter solution within 15 minutes. Repeat

this procedure until the stain is clear. Store the stain in a dark bottle in the cold. Stain remains useful for several months (McGuckin and McKenzie, 1958).

(4) 0.5% metabisulfite solution in distilled water.

(5) 3-7.5% acetic acid solution.

Procedure.

(1) Fix gel in 12.5% TCA (50 ml with gel) for 30 minutes.

(2) Rinse with distilled water overnight.

(3) Immerse in 1% Periodic acid for 30 minutes.

(4) Rinse with distilled water overnight.

(5) Immerse in Fushin Sulfite stain in dark for 50 minutes.

(6) Wash with 0.5% metabisulfite (50 ml per gel) 3x for 10 minutes.

(7) Rinse overnight in distilled water with frequent changes.

(8) When excess stain removed, store in 3-7.5% acetic acid.

Lipoprotein Stains

Sudan-Black stain (Whittaker and West, 1962). The Sudan Black solution was prepared by adding 0.1 g of Sudan Black to 100 ml of 60% ethanol and heating the solution to boiling. The hot solution was filtered twice. The solution

was cooled. The gels are placed in the freshly prepared stain for 48 hours then washed in several changes of 35% ethanol until the dark background was removed.

Sudan Black prestain (McDonald et al., 1959; Narayan et al., 1966)--Solutions of Sudan Black B were prepared by heating proplylene glycol to 100°C-110°C. (If 110°C is exceeded a gelatinous mixture results). Then adding to 100 ml of solvent, 1 g of dye. The mixture was stirred for 5 minutes. Then filtered while still hot through Whatman #2 filter paper.

After cooling the filtered solution to room temperature it was refiltered. The vitellin sample is mixed in a 2:1 ratio with the Sudan Black stain, flushed with N_2, sealed and placed at room temperature away from the light for 1 hr then transferred to a refrigerator maintained at 4° for 24 hrs. It is subsequently electrophoresed. Gels are removed and placed in 7.5% cold (2°C) acetic acid then stored under refrigeration.

APPENDIX F

COMPARISON OF MOLE PERCENTS OF AMINO ACID RESIDUES OF TWO PREPARATIONS OF A. AEGYPTI VITELLIN

Amino acid	A. aegypti vitellin (my data)	A. aegypti vitellin (H. Hagedorn)
Asx	12.7	10.6
Thr	4.3	3.8
Ser	8.8	7.7
Glx	11.9	10.7
Pro	5.3	4.8
Gly	4.4	5.5
Ala	6.3	9.3
Val	6.6	6.1
Met	1.1	2.0
Fle	4.7	3.5
Leu	6.1	6.0
Tyr	5.0	9.0
Phe	5.9	8.3
Lys	9	6.6
His	4.4	2.5
Arg	3.4	3.5

$S\Delta Q = 51.42$

This is a comparison of A. aegypti vitellin that was prepared by the lab of Dr. H. Hagedorn and analyzed on a Beckman 119C Amino Acid Analyzer by Curtis Fullmer, (Cornell University) and A. aegypti vitellin that I prepared and was analyzed on a Beckman 120C Amino Acid Analyzer by Louis Raboin (University of Massachusetts).

It should be noted that the Beckman 119C is a single column, fully automated, micro-amino analyzer, with the results being directly recorded as nMoles of each amino acid residue. The Beckman 120C is a manual, double column amino acid analyzer with the results being recorded as peaks which designate each amino acid residue. This data is then manually integrated on a Technician Manual Integrator and the nMoles of each amino acid are calculated from these results. This difference in analyzers and analyzing techniques could easily account for the variation in the results between the 2 preparations.